

Real-time Reverse Transcription Polymerase Chain Reaction Assay Panel for Middle East Respiratory Syndrome Coronavirus

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Abstract

A new human coronavirus, subsequently named Middle East respiratory syndrome coronavirus (MERS-CoV), was first reported in Saudi Arabia in September, 2012. In response, we developed two real-time reverse transcription-polymerase chain reaction (rRT-PCR) assays targeting the MERS-CoV nucleocapsid (N) gene and evaluated these assays as a panel with a previously published assay targeting the region upstream of the MERS-CoV envelope gene (upE) for detection and confirmation of MERS-CoV infection. All assays detected 10 or fewer copies/reaction of quantified RNA transcripts with a linear dynamic range of 8 log units and 1.3×10^{-3} TCID₅₀/mL of cultured MERS-CoV per reaction. All assays gave comparable performance with respiratory specimens, sera and stool spiked with cultured virus. No false positive amplifications were obtained with other human coronaviruses or common respiratory viral pathogens or with 336 diverse clinical specimens from non-MERS-CoV cases; specimens from two confirmed MERS-CoV cases were positive by all assay signatures. In June, 2012, the U.S. Food and Drug Administration authorized emergency use of the rRT-PCR assay panel as an in vitro diagnostic test for MERS-CoV. A kit consisting of the three assay signatures and positive control were assembled and distributed to public health laboratories in the U.S. and internationally to support MERS-CoV surveillance and public health response.

INTRODUCTION

On September 20, 2012, a report appeared on ProMED-mail (<http://www.promedmail.org/direct.php?id=20120920.1302733>) of a novel human coronavirus isolated several months earlier from a hospitalized patient in Saudi Arabia who had died of severe respiratory complications (1). Like the SARS-CoV, this new virus was most closely related to known bat coronaviruses, but was genetically distinct, classified phylogenetically in the group 2C coronavirus clade (2).

Subsequently named the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) for its geographic predilection (3), genomic sequence obtained from this isolate was used to develop real-time RT-PCR (rRT-PCR) assays that were released on the Eurosurveillance website on September 27, 2012(4). These assays, targeting regions upstream of the E gene (upE) for specimen screening and open reading frames (ORFs) 1b and later 1a (5)for test confirmation, have been used extensively to investigate the emergence of this new virus. As of October 4, 2013, 136 laboratory confirmed cases MERS-CoV infection, including 58 deaths, have been reported from 8 countries in the Middle East and Europe using primarily these assays (http://www.who.int/csr/don/2013_10_04/en/index.html).

On September 25, 2012, Dr. Christian Drosten at the University of Bonn Medical Center, kindly provided the U.S. Centers for Disease Prevention (CDC) sequence data from the MERS-CoV nucleocapsid (N) protein gene in advance of publication. Based on this sequence, CDC quickly developed several rRT-PCR assays targeting the N-gene to support the public health response to MERS-CoV. This report describes the validation of these assays and presents comprehensive data on the performance of the published upE assay using multiple specimen types.

103 MATERIALS AND METHODS

104

105 Viruses and clinical specimens

106 MERS-CoV strain *Jordan-N3/NCV* (2012905864/VeroP1) was kindly provided by the U.S.
 107 Naval Medical Research Unit No3 (NAMRU-3), Cairo, Egypt, with permission from the Jordan
 108 Ministry of Health (MOH). Other high-titer respiratory virus stocks or virus positive and
 109 negative clinical specimens used for assays specificity studies were available from CDC
 110 collections. Extracts from a pooled nasal wash specimen predicted to contain diverse human
 111 microbiological flora from 20 consenting healthy new military recruits were kindly provided by
 112 Dr. Lisa Lott, Eagle Applied Sciences, L.L.C., San Antonio, TX.

113

114 Three-hundred and thirty-six diverse fresh and frozen clinical specimens collected between April
 115 2011 and April 2013 from 321 persons with severe acute respiratory illness (SARI) and either
 116 resident in or with travel history to the Middle East were available for testing. Of these, 280 were
 117 combined nasopharyngeal/oropharyngeal (NP/OP) swab specimens collected in viral transport media
 118 from hospitalized Jordanian children < 2 years of age (N. Khuri-Bulos, D. C. Payne, X. Lu, D.D.
 119 Erdman, S. Faouri, A. Shehabi, M. Johnson, M. M. Becker, M. R. Denison, J. V. Williams, and
 120 N.B. Halasa, submitted for publication) with most remaining specimens from adults. A
 121 bronchoalveolar lavage sample and a serum specimen collected by the Jordan MOH Central Public
 122 Health Laboratory staff from two fatal SARI cases from a MERS-CoV pneumonia outbreak cluster
 123 at a Jordanian hospital in April, 2012, and independently confirmed positive for MERS-CoV by
 124 culture and/or sequencing by NAMRU-3, were also available for testing.

125

126 MERS-CoV Culture

127 On receipt of the virus at CDC, Vero E6 cell monolayers were inoculated and observed daily for
 128 cytopathic effect. At 3 to 4+ CPE, the cell culture lysate was recovered, aliquoted in small
 129 volumes and stored at -70°C or below. This stock virus was titered and the TCID₅₀ calculated
 130 using standard methods (stock titer, 1.3×10^4 TCID₅₀/mL). Stock virus used in spiking
 131 experiments described below was inactivated by gamma irradiation and sequence confirmed over
 132 the rRT-PCR signature regions.

133

134 Sample Processing and Nucleic Acid Extraction

For sputa or other lower respiratory specimens too viscous for downstream nucleic acid extraction, the sample was added to equal volume 500 mM freshly prepared No-WeighTM dithiothreitol (Pierce No. 20291) and incubated at room temperature for 30 min with intermittent mixing or until the sample was sufficiently liquefied for processing. For stool specimens, 10% suspensions were prepared by adding 100 μ L of liquid stool or a pea size amount of solid stool to 900 μ L of phosphate buffered saline, pH 7.4 (GIBCO), pulse vortexed for 30 sec and centrifuged at $4,000 \times g$ for 10 min at 4°C. The clarified supernatant was then carefully removed for extraction. Total nucleic acid extractions were performed on 200 μ L of sample using the NucliSens® easyMAG System (bioMérieux, Durham, NC) following manufacturer's default instrument settings and 100 μ L elution volumes were collected. For some comparison studies (see below), simultaneous extractions were also performed on the MagNA Pure Compact using Nucleic Acid Isolation Kit I (Roche Applied Science). Extracts were either tested immediately or stored at -70°C or lower until use.

Primers and Probes

Multiple primer/probe sets were designed targeting regions in the 3'-, middle and 5'-regions of the N gene sequence (GenBank accession no. JX869059.1) using Primer ExpressTM software ver. 3.0 (Applied Biosystems, Foster City, CA) predicted to specifically amplify MERS-CoV with no major combined homologies with other coronaviruses or human microflora on BLASTn analysis that would potentially yield false positive test results. All primers and probes were synthesized by standard phosphoramidite chemistry techniques at the Biotechnology Core Facility at the Centers for Disease Control and Prevention (CDC). Hydrolysis probes were labeled at the 5'-end with 6-carboxy-fluorescein (FAM) and at the 3'-end with Black Hole QuencherTM 1 (Biosearch Technologies, Inc., Novato, CA). Optimal primer/probe concentrations were determined by checkerboard titrations. Primer/probes with the highest amplification efficiencies with RNA transcripts (see below) were retained for further study (Table 1).

In vitro RNA Transcript and Viral Template Control

Single stranded DNA oligonucleotides covering the amplified region of each rRT-PCR signature and containing a 5'-T7 RNA polymerase promoter sequence (TAATACGACTCACTATAGGG) were synthesized. The oligonucleotides were amplified using the 5' T7 promoter sequence as a

forward primer and the corresponding rRT-PCR reverse primer of each signature (Table 1). Amplification products were transcribed using the MEGAscript™ High Yield Transcription Kit (Invitrogen/Life Tech). The RNA transcripts were purified using the MEGAclear Kit (Invitrogen/Life Tech) and quantified by UV light spectroscopy. MERS-CoV viral template control was prepared by combining the 3 signature templates with human genomic DNA (Promega) and then dried into a visible pellet with Pellet Paint Co-Precipitate (EMD Millipore) to create a thermostable product.

Real-time RT-PCR Assay

The rRT-PCR assay was performed using the Invitrogen SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Life Technologies). Each 25-μL reaction mixture contained 12.5 μL of 2X Master Mix, 1 μL of SuperScript® III Reverse Transcriptase/Platinum® Taq DNA Polymerase, 0.5 μL of probe, 0.5 μL each of the forward and reverse primers, 5.5 μL of nuclease-free water and 5 μL of nucleic acid extract. Amplification was carried out in 96-well plates on an Applied Biosystems® 7500 Fast Dx Real-Time PCR instrument (Life Technologies). Thermocycling conditions consisted of 30 min at 50°C for reverse transcription, 2 min at 95°C for activation of the Platinum® Taq DNA Polymerase and 45 cycles of 15 s at 95°C and 1 min at 55°C. Each run included one viral template control and at least two no-template controls for the sample extraction and reaction set-up steps. A positive test result was defined as a well-defined exponential fluorescent curve that crossed the threshold within 45 cycles. Positive viral template control (VTC) and no template controls (NTC) were included in all runs to monitor assay performance. All specimens were tested for the human ribonuclease P gene (RP) by rRT-PCR to monitor nucleic acid extraction efficiency and presence of PCR inhibitors.

RESULTS

As noted above, multiple primer/probe sets were designed targeting the MERS-CoV N gene sequence provided in advance of publication and evaluated for optimal performance with RNA transcripts. Three candidate signatures that gave the best performance, designated N1, N2 and N3, were selected for further study. However, genomic sequences obtained from clinical specimens from a Qatari patient receiving care in London in September 2012 (*England I*,

197 GenBank no. KC164505.2; *England/Qatar/2012*, GenBank accession no. KC667074.1) that
 198 later appeared on GenBank, revealed a 6 nucleotide deletion located at the 3'-end of the forward
 199 primer of N1 that would predict assay failure (Table 2). Although this deletion has not been
 200 identified again among more recently published MERS-CoV genomes, the N1 signature was
 201 withdrawn from further consideration.

202

203 **Analytical Sensitivity**

204 *Limits of detection – MERS-CoV RNA transcripts.* Serial 2-fold dilutions of each quantified
 205 RNA transcript were prepared in 10 mM TE buffer containing 50 ng/μL of yeast tRNA
 206 (Invitrogen/Life Tech) and tested by each assay signature in 24-fold replicates. The highest
 207 dilution of transcript at which all replicates were positive was defined as the limit of detection
 208 (LoD) for each assay. The LoD of all assay signatures ranged from 5 to 10 RNA transcript
 209 copies/reaction (Table 3). A linear amplification was achieved over a 8-log dynamic range from
 210 5 to 5×10^7 copies per reaction for N assays and 10 to 1×10^8 copies for the upE assay with
 211 calculated efficiency from 99.5 to 102% (Figure 1).

212

213 *Limits of detection – MERS-CoV genomic RNA.* Serial 10-fold dilutions of MERS-CoV RNA
 214 extracted from a lysate of stock cultured virus was prepared in buffer as above and tested in
 215 triplicate by each assay signature (Table 4). The LoD was approximately 1.3×10^{-3} TCID₅₀/mL,
 216 or 6.5×10^{-5} TCID₅₀ per reaction (5.0 μL/reaction), for all assay signatures.

217

218 *Limits of detection – MERS-CoV spiked in different clinical matrices.* Serial 10-fold dilutions of
 219 MERS-CoV spiked in different specimens matrices constructed from pooled human clinical
 220 samples: serum, including lipemic and hemolytic samples (10 samples); 10 NP/OP swabs in
 221 universal transport media (Diagnostic Hybrids); 10 sputum; and 15 of 10% stool suspensions as
 222 described above. The LoD of all assay signatures ranged from 1.3×10^{-2} to 1.3×10^{-3}
 223 TCID₅₀/ml across all sample matrices (Table 5). Similar results were obtained in direct
 224 comparisons between the NucliSENS® easyMAG® and MagNA Pure Compact extraction
 225 systems for NP/OP swab, serum and sputum matrices (data not shown). However, the MagNA
 226 Pure Compact was 1 to 2 logs less sensitive with stool and these results were replicated on a
 227 second instrument and using different lots of Nucleic Acid Isolation Kit I cartridges.

228

229 **Analytical Specificity**

230 *Reactivity with different MERS-CoV strains (in silico prediction).* In addition to demonstrating
 231 reactivity of the rRT-PCR assay with the MERS-CoV strain *Jordan-N3/NCV*, primer/probe
 232 sequences were evaluated against an additional 8 recently published genome sequences from 7
 233 patients collected from 6/2012 to 5/2013: GenBank accession numbers JX869059, KC776174,
 234 KC164505, KC667074, HPA Website
 235 (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317138176202), KF192507,
 236 KF186564 – KF186567. Primer/probe sequences for all signatures were 100% identical to all
 237 published virus strains.

238

239 *Cross-reactivity with other respiratory viral pathogens and human microbial flora.* The
 240 specificity of the MERS-CoV rRT-PCR assay was evaluated with purified nucleic acid obtained
 241 from a diverse collection of other respiratory virus isolates or positive clinical specimens
 242 including human coronaviruses 229E, OC43, NL63, HKU1 and SARS-CoV (Table 6). In
 243 addition to the respiratory and stool specimens described in the Clinical Studies section below,
 244 pooled nasal wash prepared from 20 healthy adults to represent diverse microbial respiratory
 245 flora was also tested. No false positive tests were obtained with any clinical sample.

246

247 **Clinical Studies**

248 *Performance of rRT-PCR assay with authentic human clinical specimens tested during*
 249 *retrospective and prospective MERS-CoV surveillance.* Results obtained with clinical specimens
 250 tested by rRT-PCR from persons hospitalized with SARI are shown in Table 7. Two specimens
 251 (1 bronchoalveolar lavage and 1 serum specimen) collected respectively from two SARI cases
 252 previously confirmed positive for MERS-CoV infection were positive by the three assays
 253 signatures. Of the 336 diverse clinical specimens from 321 other persons with SARI, all were
 254 negative by the corresponding assays. Assuming that all patients other than those associated
 255 with the Jordanian MERS-CoV outbreak cluster were not infected with MERS-CoV, the assay
 256 panel sensitivity, specificity and overall agreement were 100% (95% CI: 19.8% - 100%); 100%
 257 (95% CI: 98.6% - 100%); and 100% (95% CI: 98.9%-100%), respectively.

258

259 *Performance of rRT-PCR assay with contrived serum and stool specimens.* To obtain additional
 260 performance data from other potentially high value specimen types, 70 sera and 70 stool
 261 specimens were obtained from same number individuals with SARI or gastroenteric illness,
 262 respectively. For each specimen type, 10 randomly selected samples were spiked with moderate
 263 (1.3×10^{-1} TCID₅₀/ml) and 10 with low (1.3×10^{-2} TCID₅₀/ml) concentrations of cultured virus
 264 and 50 were left unspiked. All samples were tested blinded. Expected test results were obtained
 265 with all samples of both specimen types (Table 8).

267 **Reproducibility Studies**

268 Assay reproducibility was evaluated with three contrived respiratory specimens constructed from
 269 pooled NP/OP swab samples as above and spiked with high, moderate and low concentrations of
 270 virus. Three laboratory staff members, each on a different day and blinded to content, extracted
 271 and tested the extracts in triplicate against each assay signature. Inter-assay variation was
 272 acceptably low for all signatures (CV% range for upE, 2.01 – 4.62; N2, 2.81 – 8.09; N3, 1.96 –
 273 5.55; RP, 1.07 – 2.26) (Table 9).

275 **Test Algorithm**

276 An algorithm based on the three rRT-PCR assays was developed to guide specimen testing for
 277 MERS-CoV (Fig. 2). For routine specimen screening, N2 was combined with upE to
 278 theoretically enhance virus detection when present at low concentrations and reduce the
 279 likelihood of false negative results due to polymorphisms within the binding sites of the
 280 signature sequences. A positive test result with either or both assays would require confirmation
 281 with N3 to report a presumptive positive specimen result.

283 **DISCUSSION**

285 In response to the emergence of MERS-CoV in the Middle East and its spread to several
 286 European countries, the U.S. Health and Human Services announced on May 29, 2013, that the
 287 virus posed a significant public health threat to U.S. citizens. On June 5, the U.S. Food and Drug
 288 Administration authorized emergency use of the CDC rRT-PCR assay as an in vitro diagnostic
 289 for the presumptive detection of MERS-CoV in patients with clinical signs and symptoms of

290 MERS-CoV infection in conjunction with clinical and epidemiological risk factors
291 (<http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm>). Reagent kits
292 were distributed by the CDC Laboratory Response Network (LRN) to state public health
293 departments and to select U.S. Department of Defense surveillance laboratories equipped to
294 perform assays. The assay was also distributed to international public health partners in the
295 affected region and to countries with extensive travel to and from the Middle East.

296
297 Our assay design and validation strategy was guided by several principles. First we
298 chose to retain the upE signature designed by Corman et al. (4) due to its wide and successful use
299 in MERS-CoV surveillance. A second signature developed by these authors to confirm positive
300 upE test results that targeted the MERS-CoV 1b open reading frame (ORF) proved less sensitive
301 than upE in comparison studies (4) and was not adopted; a second assay signature was later
302 introduced targeting ORF 1a that was claimed to be as sensitive as upE(5). As an alternate
303 testing strategy, we introduced two new signatures targeting the MERS-CoV nucleocapsid (N)
304 gene; one assay (N2) was combined with upE to enhance sensitivity for specimen screening and
305 a second assay (N3) was reserved for positive test confirmation. Theoretically, rRT-PCR assays
306 targeting the MERS-CoV N gene should offer enhanced diagnostic sensitivity due to the relative
307 abundance of N gene subgenomic mRNA produced during virus replication, although we found
308 no clear evidence of this in our study nor was this shown in practice for clinical diagnosis of
309 SARS-CoV (6). Validation of all assay signatures was conducted with multiple specimen types,
310 including upper and lower respiratory specimens, serum and stool, all shown to be diagnostically
311 valuable for SARS-CoV (see below). Finally, we chose to validate the assay using instruments
312 and reagents in common use by U.S. state and international public health laboratories to
313 minimize the occurrence of off protocol use of the test.

314
315 Although the MERS-CoV rRT-PCR assay panel proved both sensitive and specific, the
316 study was subject to several limitations. First, only two authentic specimens from patients with
317 independently confirmed MERS-CoV infection were available for testing. Most data were derived
318 from mock specimens spiked with cultured virus that may not accurately replicate those obtained
319 during natural virus infection. Also, mock spiked specimens were not subjected to the same
320 collection, handling and storage conditions that authentic specimens would be subjected to that

321 might negatively impact virus detection. Moreover, we cannot be certain that the specimens
322 collected from other suspect MERS-CoV cases were truly negative for the virus. However,
323 patient demographic and clinical features, evidence of infection with other respiratory pathogens,
324 confirmed MERS-CoV seronegativity in some cases and the self-validating negative test results
325 obtained with all three assay signatures support this assumption. Finally, assay validation was
326 necessarily limited to use of specific instrumentation, reagents and procedures. Use of different
327 assay platforms or modifications in methodology could negatively impact assay performance.
328

329 The choice of appropriate specimen type, collection technique and timing from onset of
330 symptoms is also critical for diagnostic success. Among some patients, MERS-CoV appears to
331 be more often detected and with higher viral load in lower respiratory tract specimens than with
332 specimens from the upper respiratory tract (7, 8); consequently, lower respiratory tract specimens
333 have been prioritized for collection by WHO and CDC
334 (http://www.who.int/csr/disease/coronavirus_infections/update_20121221/en/). Other specimen
335 types such as sera/blood and stool may also prove valuable. Studies during the SARS epidemic
336 found that SARS-CoV could be detected in sera/blood during the early prodromic phase of
337 infection (9, 10) and in was shed for prolonged periods at high titer in stool, facilitating detection
338 later in the course of illness (11, 12). MERS-CoV RNA has been reported to be detected in the
339 stool and urine of one infected immunosuppressed patient (7) and the virus has been identified in
340 the sera of others (this study). Testing of more samples from additional patients infected with
341 MERS-CoV at all stages of illness is essential to guide testing strategies. Where respiratory
342 specimens are collected late or are not available for molecular testing, serology may be an
343 effective diagnostic alternative for MERS-CoV (13).
344

345 Even when optimal specimens and molecular tests are available, accurate diagnosis of
346 MERS-CoV infection can still be challenging. Although less susceptible to amplicon
347 contamination than conventional RT-PCR, false positive rRT-PCR tests can still occur if
348 practices designed to minimize risk of contamination are not stringently followed. Access to
349 multiple rRT-PCR assays targeting different regions of the MERS-CoV genome, with some kept
350 in reserve for positive test confirmation, is essential to prevent misidentifying MERS-CoV cases.
351 It is also recommended that laboratories conducting MERS-CoV surveillance partner with CDC

352 or another qualified reference laboratory that can independently confirm rRT-PCR positive
353 specimens by sequencing. Where rRT-PCR assays are relevant for rapid diagnosis and patient
354 management, genomic sequencing can provide public health authorities needed confidence for
355 response planning, help avoid false alarms and provide data essential to monitor both virus
356 evolution and rRT-PCR assay signature integrity.

357

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359

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Figure Legends

Fig 1. Plots of serial 10-fold dilutions ranging from 5 to 5×10^7 copies/reaction of the N2 and N3 synthetic RNA transcripts and 10^1 to 10^8 copies/reaction of the upE synthetic RNA transcripts analyzed by the MERS-CoV rRT-PCR assays. Plot inserts show calculated linear correlation coefficients (R²) and amplification efficiencies for each assay.

Fig 2. Algorithm for MERS-CoV rRT-PCR specimen testing and reporting of test results. For specimen screening, the N2 assay was combined with upE to potentially increase screening sensitivity and reduce the possibility of a false negative result due to polymorphisms near the binding site of the oligonucleotide primers or probes. A positive test result by either or both assays requires confirmation with the N3 assay to report a presumptive positive test result. VTC, viral template control; NTC, no template controls; RP, human ribonuclease P gene.

490 Table 1. Sequences of MERS-CoV rRT-PCR assay primer/probe sequences.

Assay signature	Use	Genome target	Genome location	Primers & probes ^a	Sequence (5' > 3')	50x working con.
upE ^b	Specimen screening	Non-coding region upstream envelope gene	27458-27475 ^d	Forward primer	GCAACGCGCGATTGAGTT	12.5
			27549-27530 ^d	Reverse primer	GCCTCTACACGGGACCCATA	25
			27477-27502 ^d	Probe	CTCTTCACATAATCGCCCCGAGCTCG	5
N2	Specimen screening	Nucleocapsid gene	29424-29442 ^d	Forward primer	GGCACTGAGGACCCACGTT	12.5
			29498-29477 ^d	Reverse primer	TTGCGACATACCCATAAAAGCA	12.5
			29445-29471 ^d	Probe	CCCCAAATTGCTGAGCTTGCTCCTACA	5
N3	Specimen confirmation	Nucleocapsid gene	28748-28771 ^d	Forward primer	GGGTGTACCTCTTAATGCCAATTC	25
			28814-28795 ^d	Reverse primer	TCTGTCTGTCTCCGCCAAT	25
			28773-28793 ^d	Probe	ACCCCTGCGCAAAATGCTGGG	5
RP ^c	Sample quality control	Human RNaseP gene	50-68 ^e	Forward primer	AGATTGGACCTGCGAGCG	40
			114-95 ^e	Reverse primer	GAGCGGCTGTCTCCACAAGT	40
			71-93 ^e	Probe	TCTTGACCTGAAGGCTCTGCGCG	10

^aProbes labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3'-end with Black Hole Quencher® 1 (Biosearch Technologies Inc., Novato,

^bPrimer/probe sequences from Corman et al. (2012).

^cPrimer/probe sequences from Emery et al. (2004).

^dNucleotide numbering based on human betacoronavirus 2c EMC/2012 strain (accession number JX869059.2).

^eNucleotide numbering based on human ribonuclease P (RP) mRNA (accession number NM_006413).

503 Table 2. MERS-CoV rRT-PCR assay primer/probe sequence identity with published MERS-CoV genome sequences.

Strain	Country/origin	Specimen collection date	Sample type	GenBank accession no.	Primer/probe nt sequence identity score				Reference
					upE	N1	N2	N3	
<i>HCoV-EMC</i>	Saudi Arabia	6/13/2012	Isolate	JX869059.2	100%	100%	100%	100%	2
<i>Jordan-N3/2012</i>	Jordan	04/?/2012	Isolate	KC776174.1	100%	100%	100%	100%	
<i>England 1*</i>	Qatar/Saudi Arabia	9/11/2012	Lower respiratory tract	KC164505.2	100%	6 nt deletion	100%	100%	14
<i>England/Qatar/2012*</i>	Qatar/Saudi Arabia	9/19/2012	Sputum	KC667074.1	100%	6 nt deletion	100%	100%	14
<i>England 2</i>	Saudi Arabia?	2/10/2013	Unknown	HPA Website	100%	100%	100%	100%	
<i>Munich</i>	UAE	3/22/2013	Unknown	KF192507.1	100%	100%	100%	100%	
<i>Al-Hasa_1_2013</i>	Saudi Arabia	5/9/2013	Isolate	KF186567	100%	100%	100%	100%	
<i>Al-Hasa_2_2013</i>	Saudi Arabia	4/21/2013	Isolate	KF186566	100%	100%	100%	100%	
<i>Al-Hasa_3_2013</i>	Saudi Arabia	4/22/2013	Isolate	KF186565	100%	100%	100%	100%	
<i>Al-Hasa_4_2013</i>	Saudi Arabia	5/1/2013	Isolate	KF186564	100%	100%	100%	100%	

* From the same patient

523 Table 3. MERS-CoV rRT-PCR assay limits of detection with RNA transcripts.

Predicted copies/reaction	Positive tests/24 transcript replicates (%)		
	upE	N2	N3
20	24/24 (100)	24/24 (100)	24/24 (100)
10	<u>24/24 (100)</u>	24/24 (100)	24/24 (100)
5	18/24 (75)	<u>24/24 (100)</u>	<u>24/24 (100)</u>
2.5	14/24 (58)	21/24 (87.5)	22/24 (91.7)
1.25	8/24 (33)	14/24 (58.3)	16/24 (66.7)

524 Highest dilution at which 100% of rRT-PCR replicates were positive is underlined.
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546 Table 4. MERS-CoV rRT-PCR limits of detection of culture extracted MERS-CoV RNA.

Virus quantity TCID ₅₀ /ml	upE Ct				N2 Ct				N3 Ct			
	1	2	3	Call rate ^a	1	2	3	Call rate ^a	1	2	3	Call rate ^a
1.3 x 10 ⁻⁰	27.35	27.46	27.39	3/3	27.88	27.98	28.07	3/3	25.98	25.95	25.81	3/3
1.3 x 10 ⁻¹	31.06	31.13	31.29	3/3	31.68	31.64	31.77	3/3	29.67	29.38	29.40	3/3
1.3 x 10 ⁻²	33.81	34.55	34.67	3/3	35.87	34.62	35.70	3/3	32.81	33.06	33.23	3/3
1.3 x 10 ⁻³	37.03	39.01	38.94	3/3	38.74	40.01	38.11	3/3	36.60	37.28	35.82	3/3
1.3 x 10 ⁻⁴	39.07	Neg	Neg	1/3	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3
1.3 x 10 ⁻⁵	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3
1.3 x 10 ⁻⁶	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3

^aEach assay performed in triplicate.

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565 Table 5. MERS-CoV rRT-PCR performance with different virus-spiked specimen matrices.

Virus titer, TCID ₅₀ /mL	Specimen	upE Ct				N2 Ct				N3 Ct				RP Ct			
		1	2	3	Call rate ^a	1	2	3	Call rate ^a	1	2	3	Call rate ^a	1	2	3	Call rate ^a
1.3x10 ⁰	NP/OP	26.08	26.35	26.32	3/3	25.88	25.92	25.86	3/3	23.38	23.50	23.51	3/3	27.62	27.66	27.72	3/3
	Sputum	26.62	26.72	26.75	3/3	26.07	26.13	26.15	3/3	23.69	23.74	23.76	3/3	23.43	23.56	23.61	3/3
	Serum	26.81	26.71	27.07	3/3	26.55	26.46	26.48	3/3	25.71	25.66	25.65	3/3	31.53	31.02	31.04	3/3
	Stool	27.24	27.67	28.05	3/3	28.26	28.34	28.45	3/3	25.13	25.40	25.56	3/3	37.36	38.15	36.74	3/3
1.3x10 ⁻¹	NP/OP	27.90	27.86	27.85	3/3	27.35	27.24	27.49	3/3	25.00	25.16	24.93	3/3	27.58	27.62	27.69	3/3
	Sputum	29.13	29.05	29.11	3/3	28.19	28.24	28.23	3/3	25.86	25.83	25.90	3/3	23.02	23.22	23.22	3/3
	Serum	30.18	30.76	30.75	3/3	30.11	30.26	30.23	3/3	29.30	29.26	29.37	3/3	31.19	31.04	31.42	3/3
	Stool	30.86	31.99	31.62	3/3	32.84	32.12	32.12	3/3	29.47	29.93	29.9	3/3	38	38.12	37.47	3/3
1.3x10 ⁻²	NP/OP	34.04	34.51	34.25	3/3	34.31	33.98	34.14	3/3	31.95	31.92	31.90	3/3	27.84	27.88	27.75	3/3
	Sputum	35.29	35.59	34.88	3/3	35.35	34.67	35.52	3/3	32.57	32.69	32.27	3/3	23.28	23.28	23.28	3/3
	Serum	33.62	33.67	33.54	3/3	33.03	33.00	33.33	3/3	32.70	32.43	32.18	3/3	31.29	30.98	31.19	3/3
	Stool	34.93	35.74	35.08	3/3	36.95	37.83	36.72	3/3	32.74	33.87	33.68	3/3	36.69	38.23	38.36	3/3
1.3x10 ⁻³	NP/OP	38.70	37.87	39.36	3/3	37.50	37.11	38.10	3/3	36.57	35.43	35.41	3/3	27.87	28.01	28.03	3/3
	Sputum	38.52	38.30	neg	2/3	37.58	37.94	37.50	3/3	35.49	35.44	34.90	3/3	23.21	23.25	23.27	3/3
	Serum	40.76	37.86	37.35	3/3	36.63	37.04	37.99	3/3	36.78	36.69	36.60	3/3	31.32	31.09	31.36	3/3
	Stool	39.49	neg	38.45	2/3	36.95	37.83	36.72	3/3	36.41	37.52	36.57	3/3	39.47	37.2	36.88	3/3
1.3x10 ⁻⁴	NP/OP	neg	neg	neg	0/3	neg	neg	neg	0/3	neg	neg	neg	0/3	28.01	28.00	27.96	3/3
	Sputum	neg	neg	neg	0/3	neg	neg	neg	0/3	neg	neg	neg	0/3	24.74	24.72	24.69	3/3
	Serum	neg	neg	neg	0/3	neg	neg	39.72	1/3	neg	40.44	39.58	2/3	31.40	31.28	31.65	3/3
	Stool	neg	neg	neg	0/3	neg	neg	neg	0/3	neg	neg	neg	0/3	37.6	37.24	36.99	3/3

^aEach assay performed in triplicate.

578 Table 6. MERS-CoV rRT-PCR cross-reactivity with other respiratory viruses.

Virus (strain)	Source	Other respiratory viruses rRT-PCR (Ct)	MERS-CoV rRT-PCR		
			upE	N2	N3
Adenovirus C1 (Ad.71)	Laboratory strain	Pos (13.7)	Neg	Neg	Neg
Coronavirus 229E	Laboratory strain	Pos (9.8)	Neg	Neg	Neg
Coronavirus OC43	Laboratory strain	Pos (12.9)	Neg	Neg	Neg
Coronavirus SARS (Urbani)	Laboratory strain	Pos (19.2)	Neg	Neg	Neg
Coronavirus HKU1	Clinical specimen	Pos (20.6)	Neg	Neg	Neg
Coronavirus NL63	Clinical specimen	Pos (19.9)	Neg	Neg	Neg
Enterovirus 68	Field isolate	Pos (21.3)	Neg	Neg	Neg
Human metapneumovirus (CAN 99-81)	Laboratory strain	Pos (15.0)	Neg	Neg	Neg
Influenza A H1N1 (A/India/2012)	Field isolate	Pos (14.7)	Neg	Neg	Neg
Influenza A H3N1 (A/Texas/2012)	Field isolate	Pos (10.7)	Neg	Neg	Neg
Influenza B (B/Massachusetts/1999)	Field isolate	Pos (8.4)	Neg	Neg	Neg
Parainfluenza 1 (C35)	Laboratory strain	Pos (16.6)	Neg	Neg	Neg
Parainfluenza 2 (Greer)	Laboratory strain	Pos (16.9)	Neg	Neg	Neg
Parainfluenza 3 (C-43)	Laboratory strain	Pos (15.2)	Neg	Neg	Neg
Parainfluenza 4a (M-25)	Laboratory strain	Pos (16.7)	Neg	Neg	Neg
Parainfluenza 4b (CH 19503)	Laboratory strain	Pos (21.5)	Neg	Neg	Neg
Parechovirus 1b	Field isolate	Pos (16.0)	Neg	Neg	Neg
Respiratory syncytial virus (Long)	Laboratory strain	Pos (15.0)	Neg	Neg	Neg
Rhinovirus 1A	Laboratory strain	Pos (13.4)	Neg	Neg	Neg

588 Table 7. MERS-CoV rRT-PCR results with 338 human specimens tested during retrospective and prospective MERS-CoV surveillance.
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Specimens	Specimens from MERS-CoV Confirmed Cases (Positive Results Expected)				Specimens from Other SARI Cases (Negative Results Expected)			
	upE		N2	N3	upE		N2	N3
	#	# pos/# tested	# pos/# tested	# pos/# tested	#	# pos/# tested	# pos/# tested	# pos/# tested
URT								
NP/OP swab	0	-	-	-	290	0/290	0/290	0/290
Nasal swab/wash	0	-	-	-	2	0/2	0/2	0/2
LRT								
Sputum	0	-	-	-	5	0/5	0/5	0/5
Bronchoalveolar lavage	1	1 ^a /1	1 ^a /1	1 ^a /1	20	0/23	0/23	0/23
Tracial aspirate	0	-	-	-	3	0/3	0/3	0/3
Tissue, lung	0	-	-	-	4	0/4	0/4	0/4
Other								
Serum	1	1 ^b /1	1 ^b /1	1 ^b /1	3	0/3	0/3	0/3
Stool	0	-	-	-	7	0/7	0/7	0/7
Pleural fluid	0	-	-	-	1	0/1	0/1	0/1
Urine	0	-	-	-	1	0/1	0/1	0/1
Total	2	2/2	2/2	2/2	336	0/336	0/336	0/336

^aBAL specimen: upE (Ct, 29.5); N2 (Ct, 27.9); N3 (Ct, 26.2)

^bSerum specimen: upE (Ct, 38.35); N2 (Ct, 34.9); N3 (Ct, 36.1)

599 Table 8. MERS-CoV rRT-PCR performance with individual virus-spiked serum and stool specimens.

Virus quantity TCID ₅₀ /ml	Serum sample ID	rRT-PCR assay signature			Stool sample ID	rRT-PCR assay signature		
		upE Ct	N2 Ct	N3 Ct		upE Ct	N2 Ct	N3 Ct
1.3x10 ⁻¹	SE1	32.19	32.66	31.74	ST1	34.63	33.95	32.68
	SE2	37.08	37.57	36.98	ST2	31.35	31.17	29.37
	SE3	34.10	35.02	33.88	ST3	34.43	33.51	32.48
	SE4	34.48	34.36	33.63	ST4	34.40	33.74	32.94
	SE5	36.30	36.28	35.86	ST5	33.98	33.70	32.29
	SE6	32.40	32.42	31.82	ST6	34.59	34.21	32.89
	SE7	33.28	33.28	32.60	ST7	34.07	33.43	32.16
	SE8	36.33	36.33	36.33	ST8	33.96	33.07	31.84
	SE9	33.92	34.31	33.02	ST9	32.32	32.27	30.78
	SE10	32.43	32.63	31.69	ST10	34.57	33.91	32.86
	Mean	34.25	34.49	33.76		33.83	33.30	32.03
1.3x10 ⁻²	Range	32.19 - 37.08	32.42 - 37.57	31.69 - 36.98		31.35 - 34.63	31.17 - 34.21	29.37 - 32.94
	SE11	35.56	35.93	35.78	ST11	37.08	37.10	35.89
	SE12	41.49	43.41	39.76	ST12	37.16	36.52	35.27
	SE13	36.38	37.96	38.96	ST13	37.36	36.16	35.54
	SE14	39.59	39.76	38.62	ST14	35.97	35.72	34.60
	SE15	36.47	36.12	35.75	ST15	37.76	37.10	36.50
	SE16	38.29	39.23	39.22	ST16	37.42	37.85	35.09
	SE17	38.52	38.08	38.11	ST17	36.96	37.00	36.11
	SE18	35.25	35.46	34.68	ST18	39.81	37.23	35.69
	SE19	38.18	40.11	39.23	ST19	37.53	37.39	36.00
	SE20	37.81	38.46	38.55	ST20	37.94	37.24	35.72
No virus	Mean	37.75	38.45	37.87		37.50	36.93	35.64
	Range	35.25 - 41.49	35.46 - 43.41	34.68 - 39.76		35.97 - 39.81	35.72 - 37.85	34.60 - 36.50
	SE21-70	Neg	Neg	Neg	ST21-70	Neg	Neg	Neg

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605 Table 9. MERS-CoV rRT-PCR reproducibility with virus-spiked respiratory specimen matrix.

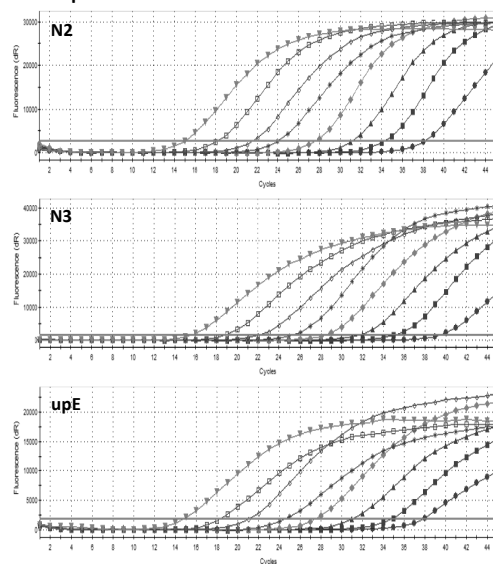
Virus titer, TCID ₅₀ /mL	upE Ct			N2 Ct			N3 Ct			RP Ct		
	1	2	3	1	2	3	1	2	3	1	2	3
Day 1, Technician 1												
3.4 x 10 ¹	20.64	20.81	20.61	19.78	19.89	19.64	18.75	18.82	18.86	33.33	33.04	32.85
3.4 x 10 ⁻¹	27.14	26.79	27.14	25.96	25.94	25.87	24.89	25.19	24.89	32.49	32.93	33.06
3.4 x 10 ⁻³	35.69	35.79	35.86	35.07	35.21	35.10	33.42	33.73	33.58	32.53	32.77	32.16
Day 2, Technician 2												
3.4 x 10 ¹	22.11	22.09	21.61	22.88	22.89	22.74	20.25	20.49	20.50	32.39	31.88	32.67
3.4 x 10 ⁻¹	28.22	27.96	28.62	29.48	29.19	29.42	27.42	27.22	27.21	32.28	33.47	33.87
3.4 x 10 ⁻³	37.59	35.32	35.30	37.63	36.74	37.02	34.38	35.23	34.52	32.12	32.32	32.71
Day 3, Technician 3												
3.4 x 10 ¹	20.68	20.56	20.47	19.92	19.28	19.68	19.18	19.21	19.24	32.34	32.26	32.68
3.4 x 10 ⁻¹	25.57	25.41	25.34	24.55	24.60	24.49	24.13	24.08	24.18	31.84	31.76	32.06
3.4 x 10 ⁻³	35.75	35.19	36.08	35.62	35.07	35.14	34.62	33.62	35.02	32.12	31.65	32.33
Summary Results	Mean	STD	CV	Mean	STD	CV	Mean	STD	CV	Mean	STD	CV
3.4 x 10 ¹	21.06	0.68	3.21%	20.75	1.58	7.63%	19.48	0.73	3.73%	32.60	0.44	1.35%
3.4 x 10 ⁻¹	26.91	1.24	4.62%	26.61	2.15	8.09%	25.47	1.41	5.55%	32.64	0.74	2.26%
3.4 x 10 ⁻³	35.84	0.72	2.01%	35.84	1.01	2.81%	34.23	0.67	1.96%	32.30	0.35	1.07%

Specimen matrix constructed from combined nasopharyngeal & oropharyngeal swabs obtained from 10 persons.

EQA panel = external quality assessment panel

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Amplification Plots



Standard Curves

